Effects of pH on Glia Proliferation and Apoptosis

Jodie Lee Kunkel

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ABSTRACT

Glioblastoma Multiforme (GBM) is the highest-grade glioma and the most malignant form of astrocytoma (Brat and Mapstone, 2003; Hjelmeland and others). Diagnosis of GBM carries a prognosis of less than 14 months after diagnosis, despite surgical intervention, radiation, and chemotherapy (Hjelmeland and others). Unfortunately, the prognosis of GBM has remained unchanged over the last thirty years, even with abundant resources devoted to research (Barres, 2008). GBM tumors have a low intracellular pH that results in increased levels of proliferation and decreased levels of apoptosis. As a result, astrocytes, the cell type responsible for GBM, have been cultured and evaluated to understand the behavior of GBM astrocytes.

Previous data shows that astrocytes cultured at pH 7.0 for several days will have higher cell numbers than those grown at the physiological pH 7.4 (Guercio, 2012). In these studies cell numbers were evaluated using manual counting to compare differences between culture conditions. A more automated approach that can evaluate multiple parameters on the same set of cells would allow for better understanding of the changes in proliferation and apoptosis in cultured cells. This would allow for more rapid turnover and consistent evaluation from culture to culture. Here we evaluated whether Promega’s ApoTox-Glo™ Triplex assay would produce reliable and repeatable results using cultured astrocytes. This assay evaluates levels of viability, cytotoxicity, and apoptosis in same set of cells.
Adult and Embryonic day 18 (E18) cells were harvested and cultured at either pH 7.0 or pH 7.4 for several days. Following the culture period, viability, cytotoxicity, and apoptosis levels were evaluated using the ApoTox-Glo™ Triplex Assay. Three different runs were completed and the results between runs varied, yet the results within runs were very consistent.

Viability or the number of live cells varied for each run of the assay with no consistent trends seen. Cytotoxicity or number of dead cells also varied between runs with E18 cells, but adult cells showed a consistent trend of increased cytotoxicity at pH 7.4. Similar to viability, apoptosis levels showed varied results but with high variability from run to run and again, no consistent trends seen.

Although the results varied from run to run, results within each run were consistent. This suggests that this assay can be used with astrocytes and that further evaluation with different toxins that change viability, cytotoxicity, and apoptosis should be used to test the sensitivity of the kit. In addition to further work up with astrocytes, GBM cell lines should be evaluated to determine if this assay could be used to evaluate changes in viability, cytotoxicity, and apoptosis with novel treatments.
Montclair State University

Effects of pH on Glia Proliferation and Apoptosis

by

Jodie Kunkel

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EFFECTS OF pH ON GLIA PROLIFERATION AND APOPTOSIS

A THESIS

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INTRODUCTION

In 1858, a scientist by the name of Rudolf Virchow wrote, “Hitherto, gentleman, in considering the nervous system, I have only spoken of the really nervous parts of it. But if we would study the nervous system in its real relations in the body, it is extremely important to have a knowledge of that substance also which lies between the proper nervous parts, holds them together and gives the whole its form in a greater or less degree” (Kettenmann and Verkhratsky, 2008; Virchow, 1871). Virchow realized the need to understand the cells in the central nervous system (CNS) that were not neurons. That substance Virchow referred to is known as glial cells. Initially thought to be filler, the discovery of glia has given way to extensive research, research that is not yet complete.

Since the discovery of glia, it has been found that they come in many varieties, serve many functions within the CNS, and outnumber neurons by 3:1 (Purves, 2012). Within the CNS, glia provide a scaffolding for neurons, guide neuronal migration, participate in synaptic transmission, and support electrical signals (Kettenmann and Verkhratsky, 2008; Ndubaku and de Bellard, 2008; Purves, 2012). Glia come in three varieties in the CNS; astrocytes, oligodendrocytes, and microglia, each of which serve different specialized functions.

Astrocytes are found only in the CNS and have a star-like appearance due to processes that protrude radially around the cell body. The function of astrocytes is to remove neurotransmitters and maintain ion concentrations at the synaptic cleft to support normal neuronal function. Astrocytes are one of few cells in the body that retain the characteristics of
stem cells which gives them the ability to generate all classes of glia found in nervous tissue (Jessen, 2004; Kettenmann and Verkhratsky, 2008; Ndubaku and de Bellard, 2008; Purves, 2012).

Oligodendrocytes, which are also restricted to the CNS, are responsible for synthesizing myelin and ensheathing the axons of neurons. Myelin helps increase the rate at which an electrical signal is propagated along the axon. Cells similar in function to oligodendrocytes exist in the peripheral nervous system. These cells are known as Schwann cells and serve the same purpose for peripheral neurons (Jessen, 2004; Ndubaku and de Bellard, 2008; Purves, 2012; Travis, 1994).

Lastly, microglia are the macrophages of the CNS. Microglia surveil the CNS and clean up any debris or waste products at sites of injury or disease (Jessen, 2004; Purves, 2012; Travis, 1994). Together astrocytes, oligodendrocytes, and microglia provide support for the central nervous system and help maintain a balanced environment for neurons.

All three types of glia work hard to maintain the CNS and facilitate optimal neuronal function. Therefore, when there is an insult to the CNS all classes of glia may become activated. Insults to the CNS include but are not limited to ischemia, hypoxia, and trauma, all of which have the ability to activate glia. If activated, oligodendrocytes will try to remyelinate any damaged axons, microglia will start to phagocytize and clean up any debris at the site, and astrocytes will begin walling off the area to minimize secondary injury (Fitch and Silver, 2008; Jessen, 2004). During the process of “walling off”, activated astrocytes can
form a dense structure called a glial scar (Fitch and Silver, 2008; Jessen, 2004). Glial scars are mixtures of astrocytes and connective tissue that serve as a space filler and act as a physical barrier to block regeneration/reconnection of affected neurons. This results in a permanent termination of communication between neurons. Unlike the CNS, neuronal injury in the peripheral nervous system can be repaired depending on the extent of damage. Some researchers believe that an insult to the CNS followed by activation of glia can lead to malignant tumors or “gliomas”, however this theory is not yet validated (Salvati and others, 2004).

Gliomas are tumors that arise from glial cells. Gliomas can arise from any of the glia described previously and the responsible cell type will be identified within the name of the tumor. The most malignant type of glioma is glioblastoma multiforme (GBM), a grade IV astrocytoma (Parsons and others, 2008). GBM tumors arise from astrocytes and are very infiltrative. Despite aggressive treatment, GBM has maintained the same prognosis for over 30 years.

When patients are diagnosed with GBM, the standard of care is resection, followed by radiation and chemotherapy. Even with this aggressive treatment the median survival time is 15 months following diagnosis (Parsons and others, 2008). Resistance to apoptosis, high angiogenicity, and/or most importantly very active proliferation all contribute to this prognosis (Sarafian and others, 2009). A combination of active proliferation followed by resistance to apoptosis can only lead to one thing a large, rapidly growing, infiltrative tumor.
Cell death can occur in one of two ways: necrosis or apoptosis. Necrosis is typically a result of trauma or injury, involves contiguous tissue, occurs rapidly, and causes inflammation. Apoptosis is programmed cell death, a process the body uses to remove unnecessary or old, worn-out cells. Apoptosis is usually only individual cells and is a carefully orchestrated series of events that result in cellular fragments that are cleared by the immune system with little to no inflammation (Steinbach and Weller, 2004; Takagi and others, 2003). Apoptosis may be triggered by multiple intrinsic or extrinsic signals and may last from hours to days depending on the cell type or types involved.

Within the CNS, cells have varying resistance to apoptosis. Neurons are the most susceptible, followed by oligodendrocytes, astrocytes, endothelial cells, and the most resistant cells being microglia (Sarafian and others, 2009). Some of the CNS signals for initiation of apoptosis in glia include an elevation in cytosolic calcium, oxidative stress, the presence of nitric oxide, mitochondrial disruption, endoplasmic reticulum stress, and protease activation (Takagi and others, 2003; Takuma and others, 2004). Of interest here is protease activation, specifically caspase activation.

Caspases (cysteine aspartate-specific proteases) are proteases that cleave an intracellular peptide at the position next to the aspartic acid residue (Creagh and Martin, 2001; Dressler and others, 2007; Takagi and others, 2003). Fourteen caspases have been identified in the apoptotic cascade (Figure 1), however there is still very little known about most of them (Creagh and Martin, 2001). Caspases are identified by numbers 1-14 and divided into two groups; initiators or effectors. Initiator caspases are the proteases.
responsible for the start or “initiation” of the apoptosis cascade, these include caspase-2, -8, -9 and -10 (Creagh and Martin, 2001; Takagi and others, 2003). Effector caspases are the proteases responsible for executing the demolition of the cell during apoptosis and include caspase-3, -6, and -7 (Creagh and Martin, 2001; Takagi and others, 2003).

Figure 1: Picture of complexity of caspase cascade (Garcia-Martin and others, 2001)
Caspases trigger cellular demolition by promoting the release of cytochrome c from the mitochondria and cleaving integral proteins within the cytoplasm and nucleus (Creagh and Martin, 2001). GBM is known to possess a resistance to apoptosis, but the mechanism is not fully understood. It is speculated that in GBM there are mutations in various parts of the apoptotic pathway that prevent activation of the cascade or halt it at certain levels, however it has not been proven (Steinbach and Weller, 2004; Ziegler and others, 2008).

The opposite of apoptosis is proliferation, or the reproduction/expansion of cells. Proliferation can occur independently or coupled with apoptosis to repair and/or replace cells. Like apoptosis, proliferation is under strict control and in most cases functions normally. Innate surveillance mechanisms will monitor and rid the body of abnormal cells, control the rate of proliferation, and monitor cell quality and quantity to prevent the formation of tumors. Unfortunately, when there is a malfunction in the control of proliferation, a cancerous tumor can result. In general, most cells in the adult brain do not proliferate or divide after development, but when triggered via trauma or insult, astrocytes can activate their stem cell properties and proliferate or generate all glia cell types in the CNS (Guizzetti and others, 2011). In the case of GBM, proliferation is up regulated, which contributes to its infiltrative properties (Sarafian and others, 2009).

Proliferation in astrocytes follows the same steps as normal cellular proliferation. Cells will first start in Gap0, followed by Gap1, Gap2, and finally enter mitosis, resulting in the generation of two daughter cells (Guizzetti and others, 2011). Usually cells require a signal or stimulus to proliferate, but GBM has the ability to enter into the cell cycle without
mitogenic stimulation (Kelly and others, 2009). Uncontrolled proliferation results in a rapid, exponential growth of cells.

Research has shown that a pH lower than the physiological pH of 7.4 increases proliferation of astrocytes in culture through the interaction of acid-sensing ion channels (ASIC) with voltage-dependent large-conductance calcium-activated potassium channels (BK) (Petroff and others, 2012). ASICs block BK channels at the physiological pH of 7.4 and in the presence of a slightly more acidic pH of 7.0 a conformational change occurs and results in relief of this inhibition (Petroff and others, 2012). There are many subtypes of ASICs, with ASIC1a being the channel found to interact with and inhibit BK channels (Petroff and others, 2012). BK channels are present in both normal and glioma cells and it has been found that their expression is positively correlated with the grade of glioma (Basrai and others, 2002; Berdiev and others, 2003; Liu and others, 2002; Ransom and Sontheimer, 2001). Targeting ASIC, BK channels or their interaction site may serve as novel method to treat GBM once the mechanisms are fully understood.

In-vitro assays exist that can evaluate multiple parameters like apoptosis and proliferation in the same set of cells, but the use of astrocytes in these assays has not been published. Here we evaluated whether Promega’s ApoTox-Glo™ triplex assay can be used to produce reliable and reproducible results using cultured astrocytes. This colorimetric assay measures viability, cytotoxicity, and apoptosis in the same set of cells using fluorescence and luminescence. In this assay, viability represents the number of live or “viable” cells; cytotoxicity represents the number of dead or “non-viable” cells; and apoptosis represents the
number of cells that are currently undergoing apoptosis. If successful, effects of different treatments on GBM cell lines in culture can be evaluated using this assay.

In order to assess the accuracy of this assay we used cultured adult and embryonic rat astrocytes, grown at either pH 7.0 or 7.4. After several days in culture, cells were evaluated for viability, cytotoxicity, and apoptotic activity using the ApoTox-Glo™ assay and results were analyzed for reliability.

MATERIALS AND METHODS

Merck Research Laboratories in Kenilworth New Jersey provided CD rats from Charles River Laboratories. All procedures using these rats were performed with permission, onsite and within established animal procedural statement guidelines approved by Merck's International Animal Care and Use Committee (IACUC).

Adult Astrocyte Culture

CD Rats, 16 weeks or older were decapitated using approved methods. Using sharp, sterile scissors, the skin along the midline of the scalp was cut and the skull exposed. Next, using sterile forceps, membranes on the skull were removed. The skull was then cut along the midline using nail nippers and carefully pried off to reveal the brain. The brain was then rinsed with chilled Hanks Balanced Salt Solution without calcium and magnesium (HBSS), and carefully lifted out of the skull using the spoon end of a sterilized weighing spatula.
Next, the brain was placed into chilled Hibernate AB (BrainBits® -HAB 500) and transported back to the culture laboratory.

In a sterile tissue culture hood, the brain was removed from the Hibernate AB, and placed on ice in a culture dish filled with clean Hibernate AB. Next, using sterile forceps, the meninges were removed from the cortex. With a sterile disposable scalpel, thin slices of the cortex were made at a 45° angle to the midline of the brain, avoiding the inner white matter. Slices were then placed into another clean culture dish on ice with Hibernate AB, and then carefully minced into smaller pieces using a new sterile sharp scissor. Minced pieces were then aspirated using a sterile Pasteur pipet and placed into a cell dissociation solution. The cell dissociation solution contained 3mL of Hibernate A without calcium (BrainBits® -HA-Ca) and 6mg papain (BrainBits® -PAP). The cell dissociation solution was prepared according to the BrainBits® papain protocol. Adult tissue was incubated in the dissociation solution for 30 minutes at 37°C and 5% CO₂ and gently swirled every 5 minutes.

After 30 minutes, tissue was removed from the papain using a new sterile Pasteur pipet and placed into 3mL of Hibernate AB. Tissue was then tritured with a silanized fire polished pipette (BrainBits® -FPP) from BrainBits® for one minute to mechanically dissociate cells. Undispersed cells were allowed to settle for one minute, and then supernatant was transferred off using a new sterile Pasteur pipet into an empty 15mL conical tube, and centrifuged at 1100rpm for one minute. The supernatant was then removed and discarded. Cells were re-suspended in 10mL of NbAstro (BrainBits® -NbAstro) and the mixture was then passed through a 40uM cell strainer. After filtration cells were again
centrifuged at 1700rpm for 5 minutes, supernatant was removed and discarded, and cells were then re-suspended in 1mL of NbAstro for counting.

Cells were counted using a hemacytometer then plated at a density of 7500 cells/cm² into BD BioCoat™ Poly-D-Lysine coated T25 flasks (#354536). Cultures were supplemented with 0.5% Gentamicin (Gibco#15750-060) to prevent bacterial contamination.

After 24 hours in culture, cells were washed with NbAstro and the media changed to remove floating debris. Debris in culture were from contamination of other cell types due to the inability to completely clean the adult rat cortices during harvesting. An example of debris in the culture is seen in figure 2A; figure 2B shows the same culture after 11 days.

Figure 2: A: Adult Cells pH 7.4, 5 days in culture. Note the debris in the flask that surrounds the cells. B: Adult cells, pH 7.4, 11 days in culture. Note how clear the culture is.

Embryonic Astrocyte Culture

Embryonic day 18 (E18) rat cortices were purchased from BrainBits® and dissociated following BrainBits’® Primary Astroglial Protocol. Cells were then plated in the same type
of plate and at the same density as adult cells. The culture protocol for the E18 cells was similar to that used for adult cells, except the incubation with papain is ten minutes and cells are not run through a strainer since embryonic tissue is still mainly undifferentiated.

**Culture Conditions**

Both embryonic and adult cells were incubated in either pH 7.4 (37°C and 5% CO₂) or pH 7.0 (37°C and 10% CO₂) until 90% confluent. Confluency was achieved 10-14 days post plating. Media was changed every three to four days in all cultures to provide ample nutrients to the astrocytes.

Once cells were 90% confluent they were harvested using 0.05% Trypsin-EDTA (Life Technologies™ 25300-054). Adult and embryonic cells were incubated with trypsin for 30 minutes to loosen their attachment to the flask. Cells were then aspirated with a serological pipette and placed into a 15mL conical tube. Cells were then centrifuged at 1100rpm for one minute then re-suspended in 1mL NbAstro and counted.

**ApoTox-Glo™ Triplex Assay**

BD BioCoat® Poly-D-Lysine 96-well plates with black sides and clear bottoms were used to run the ApoTox-Glo™ Triplex Assay. Cells were trypsinized and removed from their culture flasks and counted, then diluted to 24,000 cells/mL and 100μL of each sample was added to each well. Viability and Cytotoxicity reagents from the ApoTox-Glo™ Triplex
Assay were prepared according to manufacturer instructions then added to 96-well plate and the plate incubated at 37°C and 5% CO₂ for 1.5 hours. After incubation, the plate was analyzed for fluorescence as defined in the protocol using a Gemini XPS plate reader from Molecular Devices.

The caspase reagents were then added to the plate and the plate was incubated at room temperature for an additional 1.5 hours. Luminescence was then measured.

Figure 3: ApoTox-Glo assay overview.

The ApoTox-Glo™ Triplex Assay was run three times on three different batches of cells. The first run of this assay only included E18 cells that were in culture for 19 days. The adult cells that were co-cultured with E18 cells acquired mold contamination and were discarded and not used. Twenty replicates per condition were plated to provide a robust
sample for each group. The second run of the assay was on adult and E18 cells at both pHs after 9 days in culture to evaluate the differences between time in culture. The third and final run was on adult and E18 cells at both pHs that were in culture for 15 days. Cells were evaluated to determine if results are repeatable from assay to assay.

STATISTICS

Fluorescence and luminescence results were analyzed using a modified Excel sheet for ApoTox-Glo™ Triplex Assay from Promega’s website. Results shown have the fluorescence/luminescence of the media subtracted out.

Graphs and standard error of the mean (SEM) were generated within GraphPad Prism 5. Statistics were determined using a t-test in Excel for the first run (2 groups). Runs two and three were analyzed using a 1-way ANOVA with a Bonferroni post-test among all pairwise comparisons in Prism (4 groups).

RESULTS

Results from the first run are shown in figure 4. Cells were counted prior to plating and data is shown. This run analyzed E18 cells only. Viability and cytotoxicity readouts contained very little variability as shown by small error bars (SEM). Results showed that
cells grown at pH 7.0 grew faster than those at pH 7.4 did. There were no statistically significant differences between the two pHs for viability. However, cytotoxicity and apoptosis showed significant differences between the two pHs. Cytotoxicity showed a statistically significant difference between pHs with pH 7.4 having an increased number of dead cells per well. Apoptosis results showed a statistically significant difference that suggests wells at a pH of 7.4 contains more cells that are undergoing apoptosis.

Figure 4: Astrocytes were grown in culture for 19 days, then trypsinized and placed into 96-Well plates and assessed for Viability, Cytotoxicity and Apoptosis using Promega ApoTox-Glo™ Triplex Assay. This data suggests there are no significant differences in the number of viable cells; at pH 7.0 there are less cells that are currently dead and the caspase/apoptotic activity is also lower. This data supports previous data in suggesting the a more acidic environment inhibits apoptotic activity and decreases cell death. Proliferation is not analyzed here but may be a contributor to the survival of cells. A: Cell count using hemacytometer, showing that more cells grow at pH 7.0. B: Shows viability of embryonic cells, with no significant differences in viable/live cells between the two pH. C: Shows the cytotoxicity of embryonic cells, pH 7.0 shows a larger reduction in dead cells than that of pH 7.4. D: Shows the apoptotic activity of embryonic cells, cells grown at pH 7.0 show less apoptotic activity than cells grown at pH 7.4.
Results of the second run are shown in figure 5. This run included adult and E18 cells grown at both pHs after 9 days in culture. Cell count again showed at pH 7.0, adult and E18 cells grew faster than when grown at pH 7.4. Variability was again small within the groups. There were significant differences across all pairwise comparisons for viability and cytotoxicity. However, apoptotic activity showed no significant differences. The results of this run were not similar to the first run; however, within each condition the signals were consistent which suggests that culture period has effects on all three parameters.

![Cell Count](image1)

![Viability](image2)

![Cytotoxicity](image3)

![Apoptotic Activity](image4)

**Figure 5:** Astrocytes were grown in culture for 9 days, then trypsinized and placed into 96-Well plates and assessed for Viability, Cytotoxicity and Apoptosis using Promega ApoTox-Glo™ Triplex Assay. This data suggests there are significant differences in viability and cytotoxicity between all pairwise comparisons; and the caspase/apoptotic activity is also lower in adult cells compared to embryonic at pH 7.0, and there appears to be no difference when comparing embryonic cells at pH 7.4 to adult cells at both pH as well as comparing pH within adult cells. This data supports previous data in suggesting the a more acidic environment inhibits apoptotic activity and decreases cell death. In these graphs different superscripts representing a p<0.05 and similar letters showing no significant differences, using 1-way ANOVA with a Bonferroni post test with all pairwise comparisons. A: Shows cell count using hemacytometer, confirming that at pH 7.0 astrocytes grow faster B: Shows viability of all groups of cells to be significantly different with respect to one another. C: Shows the cytotoxicity of all groups to be significantly different from one another. D: Shows no significant differences in apoptotic activity.
Results of the third run are shown in figure 6. Adult and E18 cells at both pH were analyzed after 14 days in culture to provide a comparison to the first run. Cell counts again showed that cells grown at pH grew faster than those grown at pH 7.4 did. Variability was again minimal. Significant differences in viability were seen in all pairwise comparisons, except among adult cells. There were statistically significant differences between all pairwise comparisons in cytotoxicity. Apoptotic activity has no significant differences. Results for E18 cells showed similar trends in cytotoxicity and apoptosis when compared to run one. Results in adult cells showed similar trends in cytotoxicity only when compared to run two.

Figure 6: Astrocytes were grown in culture for 14 days, then trypsinized and placed into 96-Well plates and assessed for Viability, Cytotoxicity and Apoptosis using Promega ApoTox-Glo™ Triplex Assay. This data is similar to the first run of this assay with cells that were 19 days in culture. A: Shows cell count using hemacytometer, confirming that at pH 7.0 astrocytes grow faster. B: Viability is significantly different between all pairwise comparisons, except when comparing between pHs in adult cells. C: Cytotoxicity is significantly different among all pairwise comparisons. D: There appears to be no significant difference in apoptotic activity.
DISCUSSION

The most challenging part was establishing a harvesting protocol for adult astrocytes. This was overcome and adult cultures behaved similar to E18 cultures. Similar to previous data generated in our lab, differences in growth rates of astrocytes were seen when comparing between pHs. Astrocytes maintained at a pH 7.0 grew faster than those maintained at pH 7.4 did (Guercio, 2012). Adult astrocytes appeared to be smaller when compared to embryonic astrocytes. Culture conditions were similar to previous experiments in our laboratory, except the cells were in culture for almost two weeks before 90% confluency was achieved.

Viability was measured using a substrate that measures live-cell protease activity, GF-AFC (glycyl-phenylalanyl-aminofluorocoumarin). This substrate can only enter intact, viable cells and is cleaved by live-cell protease activity within the cell, resulting in a fluorescent signal. Each well contained the same concentration of cells, therefore it would be expected that the viability signal would not be significantly different between groups and was seen in the first run of the assay. In the second run adult and E18 cells at pH 7.0 had significant differences from those at pH 7.4. The third run showed that E18 cells had significant differences but adults did not.

Typically, the ApoTox-Glo™ Triplex Assay is run on cells in the same plate they grew in. Unfortunately, in this situation that was not possible to do this because cells needed to be in different incubators. To overcome this challenge, after their culture period cells were
cells were trypsinized and removed from their original flasks, diluted and plated so that they were all at the same density. Due to the dilution, evaluations of changes in viability using the ApoTox-Glo™ Triplex Assay are not reliable in this situation. However, it should be noted that the signals generated among wells in each group were consistent with little variability. This means that the viability readout could be used on cells that were not diluted. Differences seen here in viability are most likely due to effects of reagents from the ApoTox-Glo™ Triplex Assay on the cultured cells.

A second substrate, bis-AAF-R110 (bis-alanylalanyl-phenylalanyl-rhodamine-110) is cell-impermeant and used to evaluate cytotoxicity in wells by measuring dead cell protease activity in the extracellular fluid. Similar to the viability reagent, a fluorescent signal proportional to the number of dead-cells is generated. E18 cells in runs one and three had similar results, showing that more cells are dead at pH 7.4 than pH 7.0. E18 cells in the second run were in culture for less time than cells in runs one and three and showed more dead cells at pH 7.0 than pH 7.4. This suggests that E18 cells may be more fragile earlier in culture and culture period can cause a change in cytotoxicity regardless of pH. Adult cells were at different days in culture and results were still duplicated between the two runs, suggesting that culture period has no effect on adult cells. Results for adult astrocyte cytotoxicity showed that at pH 7.0 there are less dead cells than at pH 7.4. This data and the data from embryonic cells in runs one and three show that at a lower pH less cells die.

Evaluation of cytotoxicity will be valuable when examining the effects of compounds on cultured cells. Since the results were consistent among adult and embryonic cells at
similar days in culture, this assay is reliable for a cytotoxicity endpoint. Evaluation of this assay using a GBM cell line should be considered to investigate responses of the cells to novel therapies for treatment of GBM.

Apoptotic activity in the ApoTox-Glo™ Triplex Assay is analyzed by using a caspase-3/7 substrate. The substrate induces cell lysis, releasing any active caspases into the surrounding media. Active caspase-3/7 will cleave the substrate and generate a luminescent signal that is proportional to the amount of activity. Like cytotoxic activity, runs one and three have similar results for E18 and opposite results in run two. Adult cells did not show any significant differences among pHs in either run. Apoptosis results were repeatable when culture period was the same; further supporting that culture period has an effect. Again, these results support that this assay can be used with astrocytes.

Conclusions

Astrocytes evaluated using the ApoTox-Glo™ Triplex Assay may not have produced consistent results from run to run, but they did produce consistent fluorescence and luminescence signals within each subset of cells. This suggests that the variability may not arise from the assay itself but from actual changes in viability, cytotoxicity, and apoptosis within the astrocyte cultures. Differences may also arise from the sensitivity of cells to the reagents used, combined with an additional incubation period different from their original culture conditions. Since cells were grown in two different incubators, they were in suspension and not adhered to the 96-well plate when analyzed using the assay, which may contribute to the sensitivity of the cells and may cause variations in each run. Evaluation of
cells in an adherent culture with different toxins or compounds known to affect viability, cytotoxicity, and apoptosis may provide results that are more consistent from run to run and this evaluation should be considered in the future. An adherent culture would allow changes in viability to be assessed since cells would grow at different rates and not be diluted and normalized between wells. Here cells were plated in the 96-well plate at the same concentration in each well after determining viable cell numbers; this may have limited the resolution of the viability endpoint. It should be noted that the effects of pH on cell growth were consistent with previous data, showing that at a slightly more acidic pH, cells grow faster.

Overall, the ApoTox-Glo™ Triplex Assay is a straightforward, easy to use assay that appears to provide results with little variability within each run of the assay. In this study, cells did not receive treatments that changed viability, cytotoxicity, or apoptosis; therefore, only baseline levels of these endpoints in different culture conditions were evaluated. This may have contributed to variability in results from run to run and reduced the magnitude of changes observed in all endpoints. These results are promising and provide enough support to confirm that consistent signals are generated and changes can be evaluated using cultured astrocytes. In the future, this assay can be used to understand the effects of treatments on astrocyte viability, cytotoxicity, and apoptosis and ultimately should be used with GBM astrocytes to evaluate novel treatment for GBM.
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